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Age and Sex Related Responsiveness of *Tribolium castaneum* (Coleoptera: Tenebrionidae) in Novel Behavioral Bioassays

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ABSTRACT The hardiness and mobile nature of *Tribolium castaneum* (Herbst) make them easy to work with but are the same factors that make their responses to behavior-modifying chemical stimuli difficult to evaluate. To overcome these difficulties two bioassays were developed: a two-choice test with airflow and a diffusion-based test to evaluate responses to chemical stimuli. The two-choice assay is excellent for rapidly comparing two stimuli or examining the response to one stimulus against a control. The diffusion assay determines differences in orientation behavior to multiple simultaneous stimuli and can examine other behaviors during exposure. Preparation of individuals for bioassay is also important, because disturbance increases the activity level of individual beetles beyond the duration of the disturbance. The age and the sex of beetles affect responsiveness to chemical cues. These bioassays and a better understanding of *T. castaneum*'s activity have revealed approaches for evaluating its responsiveness to behavior-modifying chemicals.

KEY WORDS bioassay, *Tribolium castaneum*, disturbance, chemical attraction

Tribolium castaneum (Herbst) species are resistant to both starvation and dehydration and find sustenance on a wide variety of materials (Sokoloff 1974). These characteristics along with their near constant movement, especially when disturbed, make their preferences to behavior-modifying chemicals difficult to quantify. Many bioassay techniques have been used to determine the attractancy or repellency of chemical cues, but few account for the biological traits of the beetle. Previous *Tribolium* volatile assays include: a diffusion choice bioassay (Ryan and Oceallachain 1976), disk bioassay (Suzuki and Sugawara 1979, Bloch Qazi 1998), star bioassay (Pettersson 1970 [original citation], Nazzi et al. 2008), a choice test with pitfalls (Pierce et al. 1981, Boake and Wade 1984, Barak and Burkholder 1985, Mondal 1985), the walking laminar flow assay (Wood and Bushing 1963, Verheggen et al. 2007), an odor gradient olfactometer (Obeng-Ofori and Coaker 1990, Obeng-Ofori 1991), and aggregation on filter paper (Suzuki 1985). The variety of available arena designs addresses volatile perception and response questions, although it precludes any consensus on the best arena design. However, these arenas cannot fully distinguish between *Tribolium* responses due to truly directional attraction or semidirected random movement. Nonchemical cues are also important in this system, we found that visual stimuli can bring beetles close to a trap but the current chemical attractants do not lure beetles into the trap effectively (A.J.D., personal observations). In attempting to quantify differential attraction to compounds related to conspecifics, food, and shelter, we developed novel bioassays and analysis techniques.

Previous researchers have directly and indirectly addressed the differential attraction of males and female *Tribolium* to different stimuli (Keville and Kannowski 1975, Ryan and Oceallachain 1976, Oceallachain and Ryan 1977, Suzuki and Sugawara 1979, Olsson et al. 2006), mainly focusing on the aggregation pheromone,4,8-dimethyldecanal (DMD). It is a chemical that is released only by the male and shows similar attractancy for both sexes (Suzuki and Sugawara 1979). In addition, other studies have examined the relationship between chemical responsiveness and age (Dick 1937, Sokoloff 1974, Ziegler 1976) but only two paired the two to show how sex and age together relate to attraction (Arbogast and Flaherty 1973, Obeng-Ofori and Coaker 1990), showing attraction increases with age but does not vary with sex.

An effective bioassay is optimized by a thorough understanding of the importance of individual age and sex while also considering how bioassay response is related to the disturbance caused by moving the insect into the assay arena. Some bioassays describe a holding period on the arena before the bioassay starts to account for possible disturbance (Phillips et al. 1993) whereas others do not mention bioassay conditioning (Byers and Wood 1981, Nazzi et al. 2008). To test *T. castaneum* attraction to chemical cues we conducted a number of experiments to obtain baseline response rates and to determine the best way to measure the

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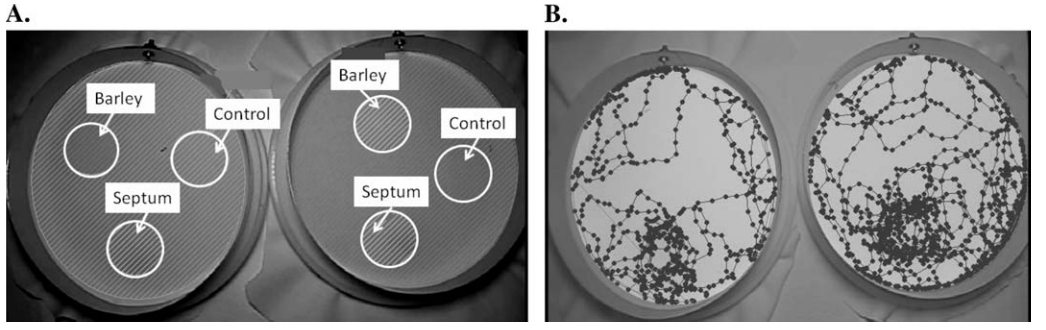

Fig. 1. A. Bioassay arena with defined zones that represent attractants located in petri dishes under a thin polyester sheet. B. A trace of the beetles movement over the arena defined above, showing a preference for the 4,8 dimethyldecanal attractant septum (Trécé).

levels of beetle response. In this study we focus on volatile attraction in the interest of developing better chemicals for beetle sampling in the future.

The following studies were conducted to develop better bioassays for monitoring the behavioral responses of *T. castaneum.* Our long-term research objectives are to develop better attractants that work effectively against beetles in field environments. In addition, the relationship between age, sex, and responsiveness has not been evaluated for food and pheromones together, and an effective bioassay to evaluate movement choices has not been agreed upon among previous researchers. Our experiments are separated into two categories: 1) response to food and conspecific cues by adult males and females as they mature, and 2) changes in activity due to disturbance.

Materials and Methods

This sequence of experiments was carried out between February and November 2009 on beetles from laboratory culture at USDA–ARS–CMAVE in Gainesville, FL. Beetles were reared on a diet of 1:1:0.4, whole wheat flour (Pillsbury, J. M. Smucker Co., Orrville, OH), medium enriched white corn meal (Dixie Mills, Tifton, GA) and brewer's yeast (MP Biomedicals, Solon, OH).

Experiment 1. The first series of experiments measured the differential levels of attraction to the aggregation pheromone, 4,8 dimethyldecanal (Trécé, Inc., Adair, OK), released from a rubber septum starting 5 min after removal from the package, a preferred food, barley, (*Hordeum vulgare* L.) (unpublished data), and a control area. Barley was freshly milled into course flour from whole kernels before the start of the experiment. Beetles were placed in a circular arena with permeable nylon fabric (50 micron mesh) pulled tight over an embroidery hoop, encircled with a 2.5-cm tall piece of aluminum flashing creating an arena 14.5 cm in diameter to contain the insects. Three plastic petri dish bottoms (3.5-cm-diameter), each containing one of the choices listed above, were placed under the fabric (Fig. 1A). One beetle was added to the arena and its movement was tracked and recorded with Ethovision XT (Noldus Information Technology, Inc., Leesburg, VA) for ten min; six to ten trials were conducted for each treatment. Trials were conducted in the dark in a Plexiglas enclosure at 26°C and 30% RH, the experiment was illuminated by an infrared LED array (Tracksys Ltd., Nottingham, UK) and recorded by an infrared video camera (Panasonic CCTV camera, WV-BP330, Pansonic Corp., Secaucus, NJ). Insects were sexed as pupae and placed into seven dram plastic vials; vials contained either beetles alone or beetles plus a small amount of diet covering the bottom of the dish, but not enough to allow the beetles to burrow. Both starved beetles and beetles taken directly from diet were tested; 2 d before each trial one group was starved for 48 h while the other was left on diet. Trials started on the first day posteclosion and continued for the first 3 wk of the insect's life. For the first 2 d of trials, the starved groups had never been on diet, but for the remaining trials all individuals had been on diet before being starved for the 48 h. Trials were conducted on insects 1–7, 10, 14, and 21-d-old.

For the first experiment, Ethovision tracked beetle location in the arena and measured time spent over the petri dishes containing barley, DMD, or an empty dish control, as well as time spent along the edges. The petri dish zones were defined by taking an image of the arena before the fabric was added, and drawing zones that corresponded to the edges of the petri dishes. When the fabric was added Ethovision recorded how much time a beetle in a trial spent over each zone. Two analyses were performed on these data, a time series analysis to determine if the movement patterns differented significantly as beetles aged (SAS 2000, Proc Mixed) and between the different treatments and an analysis of variance (ANOVA) (SAS 2000, Proc GLM) and the Tukey adjustment for multiple comparisons to see which responses among the treatments were significantly different. All statistics for this paper were generated using SAS software version 9.2 of the SAS system for Windows.

Experiment 2. To determine how *T. castaneum* move when disturbed and how movement changed over time, beetles were placed in 5.2-cm plastic petri dishes with forceps and their movement was monitored for 24 h. The experimental conditions were the same as in experiment 1. For this experiment six dishes

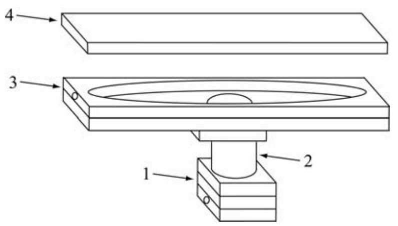

Fig. 2. The design of the oblong two-choice arena, including elements: 1) exhaust manifold, 2) introduction chamber with screen bottom, 3) arena body with two air inlets and a central outlet, and 4) a lid. The interior cutout for the arena is 17.5 cm long, 0.95 cm deep, and 5 cm wide at the widest part. The release chamber is 2.5 cm i.d. and 2.5 cm high.

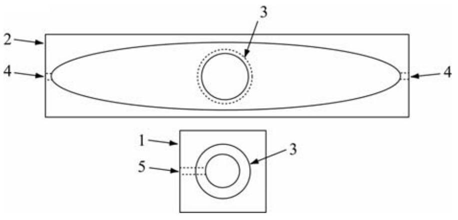

Fig. 3. A top view of the two main components of the arena, 1) the exhaust manifold, and 2) the arena body. The dotted circle three top and solid line three bottom indicate Plexiglas layers cut to the outside diameter of the introduction chamber where it fits between the two elements. Two ports four indicate where air enters the arena under positive pressure and five represents the exhaust port in the exhaust manifold.

were recorded at one time with two replicates per trial, a total of 12 replicates per treatment. There were two experimental treatments, with and without diet. Petri dishes without diet contained a circle of filter paper on the bottom of the dish to facilitate beetle locomotion, whereas dishes containing diet had a dusting of diet on the filter paper. This dusting provided food for the beetles without providing a hiding place. The dusting appeared to be a sufficient food source because it was neither completely consumed nor converted to frass over the 24-h period. Ethovision recorded insect location, sampling five times per s for 24 h and summarizing speed every 20 min for the 24-h period and every 2 min for the first 45 min. To compare movement change over time between the two different treatments, a mixed model analysis was used (SAS 2000, Proc Mixed). To compare measurements within one treatment, an unbalanced ANOVA examined the specific differences between time points (SAS 2000, Proc GLM) with the Tukey adjustment for multiple comparisons.

Experiment 3. The experiments detailed above were effective, but recording the movement of individual beetles is complicated and did not provide a straight forward choice response. We developed the following oblong two-choice arena to minimize the beetle's thigmotatic tendencies and to enable the release of multiple individuals in one trial. The arena was an oblong ellipse (17.5 cm long, 0.95 cm high, and 5 cm wide at the widest point). The purpose of the design was to eliminate corners and to minimize the time beetles spent along the edges. The arena contained a manifold and a separate insect introduction chamber so that insects introduced into the arena were not disturbed (Figs. 2 and 3). The introduction chamber was constructed from a tube of cast acrylic, 2.5 cm-i.d. cut to 2.5-cm-lengths with 53-micron Nitex fabric (Sefar AG, Heiden, Switzerland) glued on as a bottom, a lid cut from a larger Plexiglas tube with a second piece of Nitex functioning as a lid (Fig. 2).

In addition to limiting disturbance the introduction chamber ensured that the insects were exposed to air from both ends of the arena before they moved into the main arena and made a directional choice. The floor of the arena and the interior of the introduction chamber were lightly sanded to increase traction. The introduction chamber and the arena fit together so that the hole at the center of the arena had the same diameter as the i.d. of the introduction chamber (Fig. 3). Insects crawled up the sides of the introduction chamber to enter the arena. There was a second piece of Plexiglas on the bottom of the arena with a hole the size of the outside diameter of the introduction chamber to create a seal and align the introduction chamber with the arena.

The arena used a push–pull airflow system. A vacuum was connected to the manifold below the introduction chamber to pull air out while air was pushed in through both of the narrow ends of the arena (Fig. 3). The flow rates used were 38 ml/min inward from each side and a vacuum of 80.2 ml/min out through the manifold regulated with Aalborg flowmeters with carboloy floats (Aalborg, Orangeburg, NY). Between trials the inside of the arena was cleaned with ethanol and the introduction chamber was exchanged for each trial. The lid of the introduction chamber was removed before the chamber was placed into the arena and airflow was continuous through trials. This arena was tested by challenging *T. castaneum* with the DMD attractant (Trécé, Inc., Adair, OK), released from a rubber septum freshly removed from packaging on the day of the experiment. This experiment was conducted with the illumination of a red light bulb and response was recorded by visual observation. Six replicates of eight beetles were run in the arena for both male and female beetles. The number of nonresponders and number that went to each choice were recorded. The sensitivity of the new arena to detect *T. castaneum* attraction of the commercial attractant was examined with a *t*-test to determine significant attraction (SAS 2000, Proc TTEST).

Results

Experiment 1. Although *T. castaneum* were constantly moving in the arena, they demonstrated positive responses to the tested chemical stimuli by spending more time over the petri dishes containing the odor sources (Fig. 1B). An analysis of movement tracks revealed distinctly different patterns of movement over treatment and control dishes. The hierar-

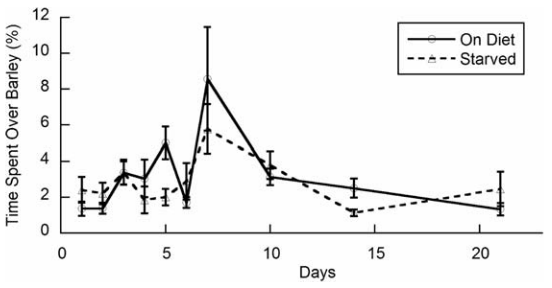

Fig. 4. The preferences of *Tribolium* that were starved and those that were removed from diet, for freshly milled barley, a preferred food source. Beetles from diet were at least as attracted as those starved to the food source, (Pearson's Correlation Coefficient = −0.042, P = 0.426, n = 355) although overall metabolic condition was a significant effect ($F_{1,998}$ = 3.89, P = 0.0489). Error bars represent standard error of the mean, n = 8.

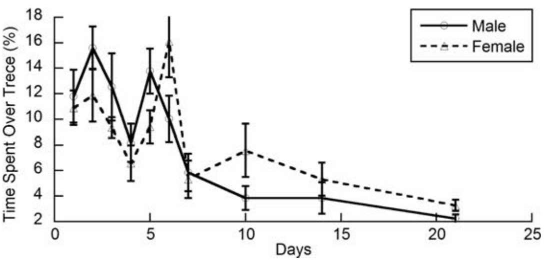

Fig. 5. A comparison of the time male and female *Tribolium* spent over the attractant septum (Trécé) emitting the 4,8 dimethyldecanal aggregation pheromone. Younger males and older females showed the strongest attraction although males and females did not show significantly different preferences (Pearson's Correlation coefficient = −0.00099, P = 0.853, n = 355). Error bars represent standard error of the mean, n = 8.

chy of variable importance by F-values in the mixed model was: sex ($F_{1,998} = 5.33, P = 0.0212$); age ($F_{9,998}$ = 4.76, $P < 0.0001$); and metabolic condition ($F_{9,998}$ = 3.90, P = 0.0485). Overall, the treatment of the zone was the most important factor but it was just the identity of the independent variable, time in zone. Movement velocity did not appear to change as beetles passed over the different zones or other areas in the arena and so spending time over chemical zones was classified as searching or attraction behavior as opposed to arrestant behavior.

The average response of starved beetles to freshly milled barley was not significantly correlated to that of beetles taken directly from diet (Pearson's Correlation Coefficient = −0.042, P = 0.426, n = 355) (Fig. 4). A time series analysis of the data demonstrated a preference shift and that overall, beetles directly from diet were more likely to spend time over the food treatment. Although metabolic condition (starved or from diet) was a significant predictor of overall time spent in zones ($F_{1,998}$ = 3.90, P = 0.049) (Fig. 4), the interaction between metabolic condition and zone treatment was not significant ($F_{2,998} = 0.58, P = 0.562$). The starving period was short compared with *T. castaneum*'s ability to go without sustenance (Sokoloff 1974) and so the similarity in preference was not surprising. Beetle sex was also a significant predictor of the time spent in zones ($F_{1,998} = 5.33, P = 0.021$) but it did not significantly interact with zone treatment ($F_{2,998}$ = 1.76, P = 0.173). A further examination indicated gender was not significantly related to time spent over the pheromone zone (Pearson's correlation coefficient = −0.0099, $P = 0.853$) (Fig. 5). Beetle age by chemical stimuli was the only significant interaction between variables, ($F_{18,998}$ = 4.78, P < 0.0001) indicating preferences for specific chemical stimuli change significantly as beetles age. The lack of significance in the other treatments by dependent variable interactions shows metabolic condition and sex were not related to the time spent over specific zones, expected to be barley and 4,8-dimethyldecanal respectively. An ANOVA over all ages and conditions indicated that beetles spent significantly more time over barley ($F_{1,998}$ = 29.92, P < 0.0001) and DMD ($F_{1,998} = 286.13, P < 0.0001$) than the control and that DMD was significantly preferred over barley ($F_{1,998}$ = 130.27, P < 0.0001) (Fig. 6).

Experiment 2. In the examination of behavior change after disturbance, beetles moved more rapidly after they were disturbed and added to an arena (Fig. 7). Although their movement slowed over time, it took longer than 2–10 min usually employed in experimental designs. When placed on diet it took ≈4 h for *T. castaneum* to slow down to a basal activity level (Fig. 7) although only the first three 20 min periods were significantly different from most of the other time points (Tukey–Kramer, P < 0.0001, df = 71). When placed in an arena with filter paper without food, the beetles calmed down twice as fast, but were more variable in their responses and beetles never slowed like those on diet ($F_1 = 95.30, P < 0.0001$) (Fig. 7). On filter paper *T. castaneum* moved over a significantly

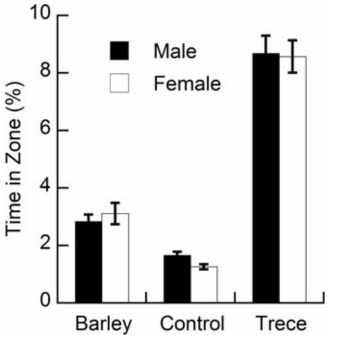

Fig. 6. A comparison of the overall average times spent by males and females over the various stimuli. Beetles spent more time over the aggregation pheromone than barley ($F_{1,998}$ = 130.27, P < 0.0001) but both zones received more attention than the control, barley ($F_{1,998} = 29.92, P < 0.0001$) and DMD septum (Trécé) ($F_{1,998} = 286.13, P < 0.0001$). Time spent by gender among the choices were not significantly different ($F_{2,998}$ = 1.76, P = 0.173). Error bars represent standard error of the mean, n = 8.

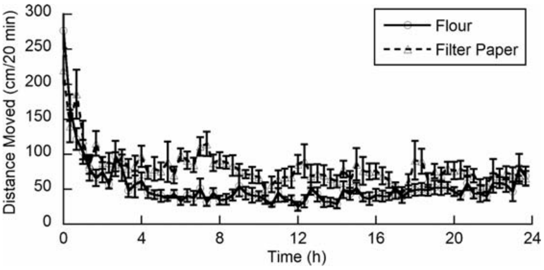

Fig. 7. The change in the movement of *Tribolium castaneum* over 24 h in an arena either with a thin dusting of flour or filter paper only. The beetles move more slowly over time when they have food and more erratically when they do not, their movement rates on the different substrates are significantly different ($P < 0.0001$). Error bars represent standard error of the mean, $n = 12$ per substrate.

greater distance during the first hour but was not different from the long-term average after that point (Tukey–Kramer, $P < 0.05$, df = 71). The use of ANOVA on individual points did not encompass the linear trend of decreasing activity and so trends were also analyzed with mixed models. Both treatment ($F_1 = 95.30$, $P < 0.0001$) and time ($F_{71} = 9.45$, $P < 0.0001$) were highly significant variables in this analysis although the trends over time (curve shapes) were not significantly different between treatments ($F_1 = 1.21$, $P = 0.121$).

Experiment 3. The two-choice arena was an effective design to test the preferences of male and female *T. castaneum* to the DMD attractant septum (Fig. 8). The assay showed that males and females were both attracted to the septum ($t_{22} = 4.29$, $P = 0.0003$) but did not respond in significantly different numbers ($t_{22} = 1.00$, $P = 0.0326$).

Discussion

The objectives of this study of *T. castaneum* were to use its ecology as a fundamental tool for the creation of better attractants. Therefore the development of better assays and experimental techniques that not only demonstrated significant attraction, but also enticed *T. castaneum* to leave food materials and move toward another cue were necessary. As a first step in this process past experiments that addressed the question of attraction were examined. Young beetles are more likely than older beetles (>10 d as adults) to emigrate in search of fresh habitat (Ziegler 1976, Ziegler 1978) although they are less likely to venture into light (Arbogast and Flaherty 1973) while remaining more responsive to aggregation pheromones. This study shows that time spent over barley and a control area did not change significantly with age, thus demonstrating that throughout their early lives *Tribolium* do not change their food preferences, although they do start out more likely to disperse in search of mates, conspecifics and to some degree food. As they aged, even those that had not mated spent less time around chemical cues, although they still responded significantly.

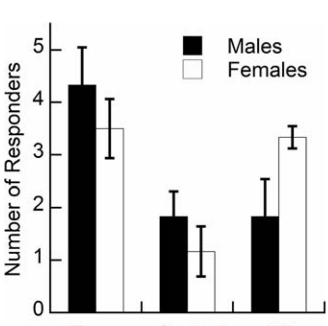

Fig. 8. The response rate of newly emerged unfed and fed beetles to the DMD septum (Trécé) in the oblong two-choice arena. This is a summary of ten trials for each sex with eight beetles per trial.

T. castaneum is most likely to spend time in flour patches, and when outside of patches, to spend time along edges and in corners (Campbell and Hagstrum 2002). The relatively small amount of time spent above attractive substances in the central part of an arena is likely due, in part, to this tendency to remain along edges. The Ethovision analysis demonstrates significant differences between time spent over the different choices and the control area. The decreased response to aggregation pheromones over time could be related to mating and dispersal considerations including early dispersal from larval habitat and a search for unoccupied resources. This contrasts with previous research that indicates that maximum responsiveness occurs after 19 d for males and 21 d for females (Obeng-Ofori and Coaker 1990). Altered responses could be linked to preparation of individuals for bioassay, Obeng-Ofori and Coaker (1990) aged beetles individually while this study held them in same sex cohorts. Because female and male responses to pheromones decline similarly, habituation could not be the only factor for females who do not produce 4,8-dimethyldecanal.

The only significant interaction between the treatments, age, sex, and metabolic condition with zone stimuli is age. Indicating that time spent in specific zones changes with age across all other treatments. The strongest factor is the decline in the time spent by beetles prepared in this manner over 4,8-dimethyldecanal and this pattern overshadows any other patterns in the data.

The movement decay experiment demonstrates that it is very important to consider the disturbance effects on *Tribolium* movement and response. The increase in activity level caused by disturbance before bioassay lasts longer when beetles are added to an arena with food. Without food, beetles move faster on average, but the initial movement rate is lower and differs less from the steady state level reached after a few hours. While one hour of rest will move beyond the time points where activity was significantly increased above the other time points, it is important also to consider the trend that indicates a slightly longer period to reach basal activity levels. Movement is more directed and faster when the beetles are exposed to aggregation pheromones (Obeng-Ofori 1991), and similarly, initial movement and search is more rapid when on diet but once the insect recovers from the disturbance their basal movement rate is both lower and less variable.

Our novel bioassay chamber solves the problem of insect disturbance and also addresses a rapid screening of different compounds and the separation of nonresponders in the evaluation of attraction. With a separate insect introduction chamber that becomes part of the choice chamber we are able to introduce small insects without manipulating them. The two-choice design is similar to some y-arm olfactometer assays, but has the added benefits of allowing the insect to move from the center to make a choice, while keeping them from following walls into corners or being unduly directed toward one choice or another. Future experiments will take into account both disturbance effects and age because both strongly influence behavior.

Acknowledgments

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